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1. Streamer KE et al. J. Biol. Chem, 1989, Vol. 264(10):5331-5334.
2. Stafforini DM et al. J. Biol. Chem., 1993, Vol. 268(6):3857-3865.
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# The Platelet-activating Factor Acetylhydrolase from Human Erythrocytes

## PURIFICATION AND PROPERTIES\*

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Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a biologically active phospholipid. Tissues, blood cells, and plasma contain PAF acetylhydrolases (calcium independent phospholipase A<sub>2</sub> activities) that catalyze the hydrolysis of phospholipids containing short chain *sn*-2 acyl groups. They inactivate PAF and thereby determine PAF accumulation.

We purified the PAF acetylhydrolase from human erythrocytes 15,600-fold. The enzyme has a molecular weight of 25,000, it behaves as a dimer during gel filtration, and it is a previously uncharacterized cytosolic esterase, as it has a unique amino-terminal sequence.

The erythrocyte PAF acetylhydrolase requires the addition of sulfhydryl agents for maximal activity, is inhibited by 5,5'-dithiobis(2-nitrobenzoic acid), NaF, diisopropyl fluorophosphate, diethylpyrocarbonate, *p*-bromophenacylbromide, and a number of proteases. Antibodies against the purified protein precipitate all PAF hydrolase activity from erythrocyte lysates.

The erythrocyte PAF acetylhydrolase is specific for short or oxidized *sn*-2 acyl residues. It exhibits surface dilution kinetics, suggesting that hydrolysis occurs at lipid interfaces. This suggests that this enzyme acts *in vivo* as a scavenger of oxidatively fragmented phospholipids that are toxic to the cell.

Platelet-activating factor (PAF,<sup>1</sup> 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a biologically active phospholipid that stimulates platelets, neutrophils, and other proinflammatory cells. PAF mediates a wide spectrum of actions including hypotension, changes in smooth muscle tone, increased vascular permeability, and stimulation of glycogenolysis (1). PAF is produced by many types of cells and tissues (2-5). It is thought to be an important signal in inflammation and allergy and in the nervous (6) and reproductive (7) systems. In addition, a family of phosphatidylcholines that are structurally related to PAF has been identified in the lipid

extracts of bovine brain (8-10). These compounds have diverse *sn*-1 acyl residues and a series of short aliphatic dicarboxylic (9) or monocarboxylic acid residues (8) at the *sn*-2 position. Interestingly, they are toxic, induce hypotension, and mimic other actions of PAF, including platelet aggregation. The formation of vasoactive choline phosphoglycerides may result from chemical oxidation and fragmentation of polyunsaturated fatty acyl residues. For example, we have shown that oxidative fragmentation of 1-palmitoyl-2-arachidonoyl-glycerophosphocholine generates diacylphosphatidylcholines with a spectrum of short to intermediate length *sn*-2 residues (11). These oxidatively fragmented phospholipids are metabolized by removal of the *sn*-2 acyl group, and this reaction is catalyzed by the plasma PAF acetylhydrolase (12). These compounds are biologically active (13) and, in addition, are hydrolyzed with catalytic efficiencies comparable to those observed with PAF as a substrate (12). Thus, the role of the plasma PAF acetylhydrolase is broader than previously thought since, in addition to PAF, this enzyme has the ability to catalyze the hydrolysis of oxidatively fragmented phospholipids.

The synthesis of PAF has been examined in a variety of mammalian cells, and it is known to be tightly regulated (14). Its degradation is catalyzed by a specific acetylhydrolase, which removes the acetyl group of PAF to produce the biologically inactive lyso-PAF (15, 16). This process can also regulate PAF accumulation in certain cell types (17-20). PAF acetylhydrolase is present in mammalian tissues and blood (21), and changes in the level of activity in plasma have been observed in human diseases, suggesting that the rate of degradation of PAF also may be a mechanism to regulate its physiological effects (22-24). We purified the PAF acetylhydrolase from human plasma to near homogeneity (25), showed that it is highly selective for a short acyl residue at the *sn*-2 position, described its association with lipoproteins (26), and examined its role in the degradation of PAF in plasma (27).

In addition to the extracellular plasma enzyme, intracellular PAF acetylhydrolase activity is present in many mammalian cells and tissues (21, 28). We and others have reported the occurrence of a family of PAF acetylhydrolases, both intra- and extracellular, that share similar properties, but that can be differentiated from one another by a variety of criteria (21, 29). None of the intracellular enzymes have been purified or characterized to date. The intracellular PAF acetylhydrolase regulates the accumulation of PAF under some circumstances: for example, in macrophages (17, 18) and platelets (19, 20) when synthesis is maximally stimulated, the amount of PAF that accumulates at any time is also determined by the activity of PAF acetylhydrolase. Our goal is to eventually define the

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† Established Investigator of the American Heart Association along with Dr. Zimmerman while much of this work was performed.

<sup>1</sup> The abbreviations used are: PAF, platelet-activating factor; PAGE, polyacrylamide gel electrophoresis; DTE, dithioerythritol.

role that the intracellular PAF acetylhydrolases play *in vivo*, to determine whether they are related to each other, and to examine their structural and functional properties. We chose human erythrocytes as the starting material to purify an intracellular PAF acetylhydrolase because these cells can be isolated in large quantities and they have high levels of the enzymatic activity (30–32). In this paper, we report the purification and characterization of the intracellular PAF acetylhydrolase activity from human erythrocytes, and we propose that its function is to protect erythrocytes from damage caused by lipid peroxidation.

## EXPERIMENTAL PROCEDURES

### Materials

All phospholipid substrates were obtained from Avanti Polar Lipids. [acetyl-<sup>3</sup>H]PAF (specific radioactivity = 10 mCi/μmol) and 1-O-[alkyl-<sup>3</sup>H]PAF (specific radioactivity = 10–30 mCi/μmol) were purchased from Du Pont-New England Nuclear. 1-Palmitoyl-2-[(1-<sup>14</sup>C]5-oxovaleryl)-sn-glycero-3-phosphocholine and 1-[1-<sup>14</sup>C]palmitoyl-2-valeryl-sn-glycero-3-phosphocholine were synthesized as previously described (11, 12). DEAE-Sepharose CL-6B and Sephacryl S-300 were from Pharmacia LKB Biotechnology Inc. Octadecyl silica gel cartridges were from Baker Chemical Co. Affi-Gel 501 was from Bio-Rad. Protein A-Sepharose 6MB and other reagents were from Sigma.

### Enzyme Assays

PAF acetylhydrolase activity was assayed as previously described (25), except that the assays were supplemented with dithiothreitol or dithioerythritol (0.2 mM final concentration in the assay). 2,3-Bisphosphoglycerate mutase activity was determined using the spectrophotometric assay of Sasaki *et al.* (33).

### Purification of the Human Erythrocyte PAF Acetylhydrolase

**Cell Isolation**—Human erythrocytes were isolated from freshly drawn human blood (800 ml) from healthy volunteers. The blood was collected in EDTA (25 mM) and then centrifuged for 20 min at 2,000 × *g*. The supernatant was discarded, and the blood cells were suspended in 3 volumes of 0.9% NaCl and centrifuged as above. The straw-colored supernatant was discarded, and the cells were washed twice more to remove contaminating plasma PAF acetylhydrolase. The buffy coat remaining on top of the red blood cells was discarded. In preliminary experiments, we found that the amount of PAF acetylhydrolase activity associated with the white blood cells was much smaller than that associated with the red blood cells. Thus, contamination with white blood cells (if any) would contribute little to the total erythrocyte activity. To the erythrocyte preparation we added aprotinin (4.8 trypsin inhibitor units), NH<sub>4</sub>Cl (33.5 g), DTE (10<sup>-3</sup> moles), EDTA (4 × 10<sup>-3</sup> mol), and water to a final volume of 4 liters. The preparation was stirred overnight at 4 °C to lyse the erythrocytes. All chromatography was carried out at 4 °C.

**Batch and Column DEAE-steps**—A slurry of DEAE-Sepharose CL-6B (approximately 400 ml) in 5 mM sodium phosphate buffer (pH 6.8) containing 1 mM EDTA and 250 μM DTE was added to the lysed erythrocyte preparation and was stirred at 4 °C for 6 h. The slurry was packed in a column (5 × 90 cm), the effluent was collected, and the gel was washed with equilibrating buffer until virtually no protein eluted from the resin (1–1.5 liters of buffer). This step removed most of the hemoglobin, which is one of the major proteins in this preparation. The PAF acetylhydrolase was eluted from the gel by treatment with four 250-ml washes of 0.3 M KCl in equilibrating buffer. Most of the activity was present in the third wash, which was diluted to 2,500 ml with 5 mM sodium phosphate buffer (pH 6.8) containing 1 mM EDTA and 250 μM DTE. The preparation was then loaded on a DEAE-Sepharose CL-6B column (2.5 × 60 cm) and equilibrated in the same dilution buffer, at a flow rate of 70 ml/h. The column was washed with 500 ml of equilibrating buffer at a flow rate of 95 ml/h. Next, we applied a 500-ml linear gradient (0.1–0.4 M KCl in buffer), followed by a 200-ml wash with 0.4 M KCl in buffer, at a flow rate of 35 ml/h. Fractions (6 ml) were collected and assayed for protein content and PAF acetylhydrolase activity. Active fractions were pooled and concentrated to 20 ml by ultrafiltration using a PM-10 filter (Amicon Corp.).

**Sephacryl S-300 Step**—The pooled, concentrated fraction from the

column DEAE-step was placed on a Sephacryl S-300 column (2.5 × 90 cm) equilibrated in 10 mM sodium phosphate buffer (pH 6.8) containing 250 μM DTE and 1 mM EDTA, at a flow rate of 19 ml/h. Fractions (5 ml) were collected and assayed for protein content and PAF acetylhydrolase activity. Active fractions were pooled and concentrated to 17 ml using ultrafiltration as above.

**Native Polyacrylamide Gel Electrophoresis**—To the concentrated S-300 effluent we added sucrose and bromphenol blue to final concentrations of 10% and 0.0005%, respectively. The preparation was placed on two native polyacrylamide (7%) gels (3-mm thickness) and subjected to electrophoresis at 40 mA until the dye reached the bottom of the gels. The gels then were sliced horizontally into 22 fragments, which were forced individually through a syringe and rocked overnight at 4 °C in 5 ml of 10 mM sodium phosphate buffer (pH 6.8) containing 250 μM DTE. The eluate from the slices was recovered by centrifugation and assayed for PAF acetylhydrolase activity and protein content. The active fractions were pooled and dialyzed overnight against 20 mM Tris-HCl buffer (pH 7.5).

**Mercurial Agarose Column**—We next placed the dialyzed preparation (23 ml) on a mercurial agarose column (0.9 × 15 cm) equilibrated in 20 mM Tris-HCl buffer (pH 7.5), at a flow rate of 13.3 ml/h. The column was washed with 50 ml of the equilibrating buffer. At this point the flow of buffer was reversed to minimize nonspecific binding of the enzyme to free resin, and then a 100-ml linear gradient of DTE (0–5 mM) was applied at the same flow rate. The active fractions (2 ml each) were combined, and NaCl was added to a final concentration of 1.5 M.

**Phenyl-Sepharose Column**—The preparation was next placed on a phenyl-Sepharose column (0.9 × 15 cm) equilibrated in 20 mM Tris-HCl (pH 7.5), containing 1.5 M NaCl and 1 mM DTE, at a flow rate of 13.5 ml/h. The column was washed with 45 ml of equilibrating buffer, and then a 200-ml linear gradient of NaCl (1.5 M–0 M in buffer) was applied to the column at the same flow rate. Fractions (3.4 ml) were collected, and those containing PAF acetylhydrolase activity were pooled and concentrated using ultrafiltration, as above.

### Antibodies and Immunoprecipitations

Polyclonal antibodies against the erythrocyte PAF acetylhydrolase were raised in New Zealand White rabbits following the procedure of Vaitukaitis (34). The IgG fraction was isolated by treatment with caprylic acid, as described. Immunoprecipitation assays were performed by incubating a source of the antigen with the IgG fraction of preimmune or immunized rabbits for 1 h at 37 °C, in a total volume of 50 μl. Then, protein A-Sepharose 6MB (25 μl of a 50:50 suspension) was added, and the incubations were continued for an additional hour at 37 °C. The supernatants of these incubations were carefully removed and assayed for PAF acetylhydrolase activity. The beads were washed three times with 1 ml of phosphate-buffered saline and then assayed for activity.

### Sequencing

The two products of the purification of the erythrocyte PAF acetylhydrolase were sequenced by Dr. Robert Shackmann at the Protein-DNA Core Facility of the University of Utah. This was accomplished using a protein microsequencer (model 477A, Applied Biosystems), using Edman chemistry. The amino-terminal residue of the 31-kDa protein (see below) was blocked, and it was therefore necessary to fragment this protein product to obtain internal sequence. To accomplish this, a sample (6.5 × 10<sup>-4</sup> μmol) was treated with CNBr (7.5 μmol) in 80% formic acid for 18 h at 25 °C in the dark. The sample was dried under N<sub>2</sub> and resuspended in 0.1 M Tris-HCl buffer (pH 7.0). Before sequencing, the 25-kDa and the CNBr-treated 31-kDa products were subjected to electrophoresis on 10% SDS-PAGE gels and then transferred to a polyvinylidene difluoride membrane (Applied Biosystems). The proteins were visualized by staining with Coomassie Brilliant Blue, sliced, and submitted to microsequencing.

### Assay of PAF Synthesis

Suspensions of washed erythrocytes were incubated in Hanks' balanced salt solution with 10 mM Ca<sup>2+</sup>, 100 μCi of [<sup>3</sup>H]acetate (Du Pont-New England Nuclear, 50 mCi/μmol) and calcium ionophore A23187 (10 μM), or buffer alone for 10 min, in a total volume of 1 ml. The assay was stopped and the production of PAF was assessed as previously described (2). This assay is highly sensitive and will measure the synthesis of all acetylated phospholipids, regardless of the linkage at the *sn*-1 position. Identical incubations were carried

out in parallel using endothelial cells or neutrophils as positive controls.

# RESULTS

**Purification of the PAF Acetylhydrolase from Human Erythrocytes**—We first tested the location of the PAF acetylhydrolase in lysates of erythrocytes. We found that 96% of the activity was in the soluble fraction, in agreement with another report (31). This fraction was used for subsequent purification. The elution profiles from DEAE-Sephacryl CL-6B, Sephacryl S-300, native polyacrylamide gel electrophoresis, Affi-Gel 501, and phenyl-Sepharose are shown in Fig. 1. The summary of the purification is shown in Table I. The overall

purification was 15,600-fold, and the recovery was 7%. In recent studies, we have found that the recovery from the native polyacrylamide step can be improved to 100% recovery of activity by supplementation of the stacking and running gels and the running buffer with 250  $\mu$ M dithiothreitol. We evaluated the product of our purification protocol by SDS-PAGE and detected two bands (molecular weights 25,000 and 31,000) by silver staining (Fig. 2). When we examined the proteins at various stages of the preparation we found that the intensity of these bands increased as the preparation was enriched in activity. We identified the 31,000-Da band as 2,3-bisphosphoglyceromutase by sequence analysis (see below). Thus, the PAF acetylhydrolase activity is likely the 25,000-

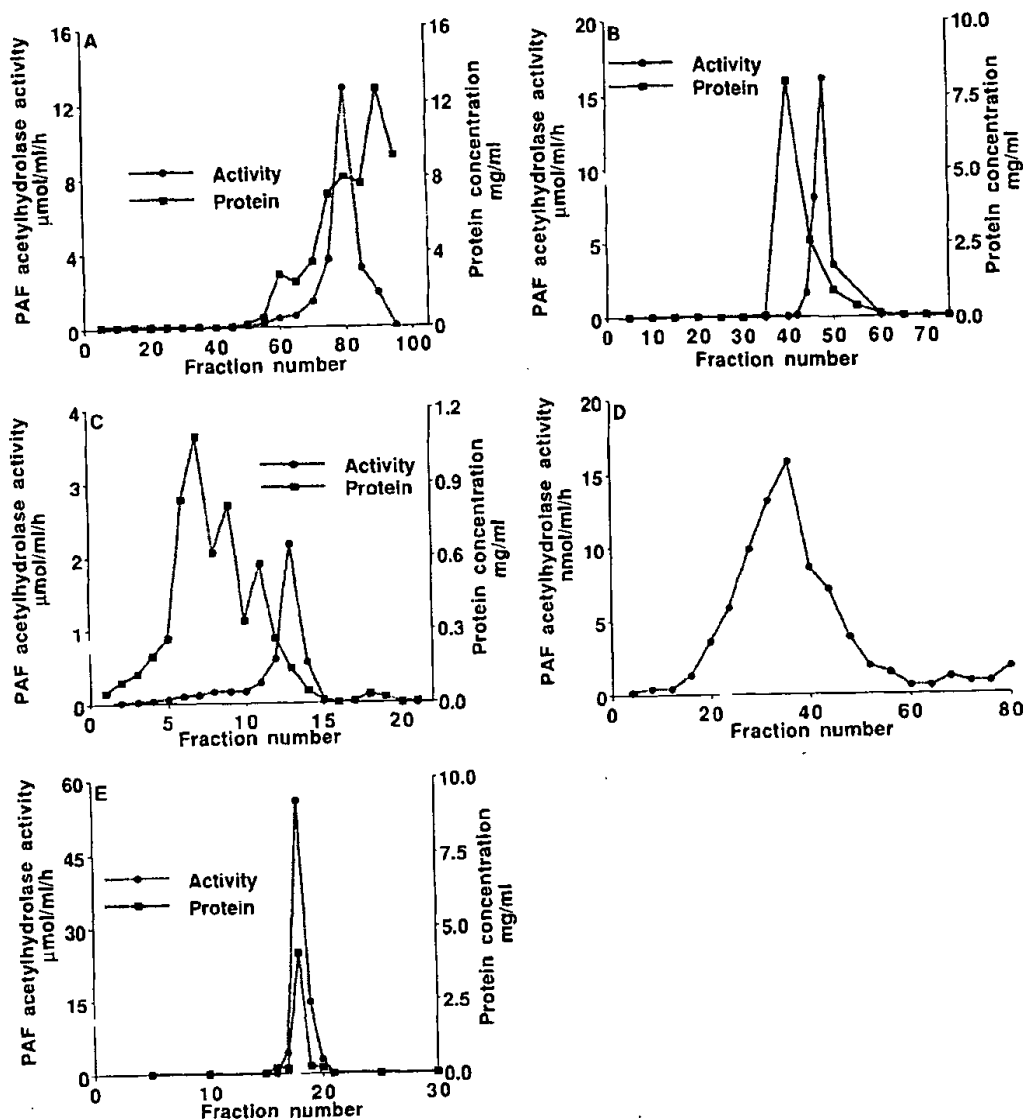


FIG. 1. Purification of human erythrocyte PAF acetylhydrolase. A, DEAE-Sephacryl CL-6B. The effluent from the batch DEAE-Sepharose (1000 ml) was placed on a DEAE-Sephacryl CL-6B column, as described under "Experimental Procedures." Fractions (6 ml) were collected and assayed for PAF acetylhydrolase activity and protein content. B, Sephacryl S-300. The concentrated effluent from the DEAE-Sephacryl column was placed on a Sephacryl S-300 column that had an exclusion volume of 194 ml and a total volume of 442 ml. Fractions (6 ml) were collected and assayed for PAF acetylhydrolase activity and protein content. C, native polyacrylamide gel electrophoresis. The Sephacryl S-300 effluent was concentrated and placed on two native polyacrylamide gels. After electrophoresis, the gels were sliced, and the proteins were recovered from individual slices and then assayed for PAF acetylhydrolase activity and protein content, as above. D, phenyl-Sepharose. The effluent from the native PAGE step was placed on a phenyl-Sepharose column, as described under "Experimental Procedures." Fractions were collected and assayed for PAF acetylhydrolase activity. E, organomercurial agarose. The effluent from the phenyl-Sepharose step was placed on an organomercurial column, as described under "Experimental Procedures." Fractions were collected and assayed for PAF acetylhydrolase activity.

TABLE I  
 Purification of human erythrocyte PAF acetylhydrolase

| Fraction              | Units             | Protein           | Specific activity | Recovered | Purification |
|-----------------------|-------------------|-------------------|-------------------|-----------|--------------|
|                       | $\mu\text{mol/h}$ | $\text{mg}$       | $\text{units/mg}$ | %         | -fold        |
| Red blood cell lysate | 620               | $2.0 \times 10^5$ | 0.0031            | 100       |              |
| Batch DEAE-effluent   | 671               | $1.6 \times 10^5$ | 0.42              | 108       | 135          |
| Column DEAE-effluent  | 859               | 562               | 1.53              | 138       | 494          |
| Sephacryl S-300       | 666               | 255               | 2.6               | 107       | 842          |
| PAGE                  | 105               | 12                | 8.4               | 16        | 2,710        |
| Hg-agarose            | 115               | 6.5               | 17.8              | 19        | 5,742        |
| Phenyl-Sepharose      | 41                | 0.8               | 48.3              | 7         | 15,600       |

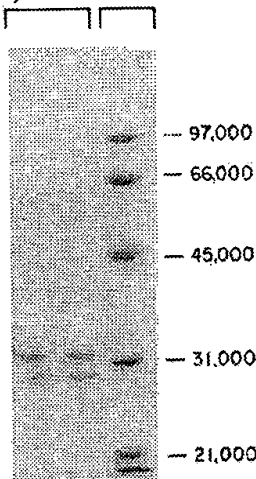
 Purified Erythrocyte  
 PAF Acetylhydrolase MW Standards


FIG. 2. SDS-polyacrylamide gel electrophoresis of the purified erythrocyte PAF acetylhydrolase. Lane 1, molecular weight markers; lanes 2 and 3, purified product.

Da component. The purified enzyme is stable for at least 1 year when stored in frozen form at pH 7.5 in the presence of a reducing agent. The results of this purification scheme are representative of three independent runs.

**Characterization of the Substrate Specificity of the Erythrocyte PAF Acetylhydrolase**—One remarkable feature of the plasma PAF acetylhydrolase is its marked selectivity for phospholipids with short acyl chains at the *sn*-2 position; activity falls to unmeasurable levels with chains above 5 carbons in length (11). We asked whether the erythrocyte enzyme had the same specificity. We examined the ability of the purified enzyme to catalyze the hydrolysis of 1-stearoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine and found that this compound was not hydrolyzed (not shown). Next, we tested the effect of various PAF analogs for their ability to inhibit PAF hydrolysis (Table II). Compounds containing short acyl groups at the *sn*-2 position inhibited PAF hydrolysis by the purified enzyme. These results demonstrate that a short *sn*-2 acyl substituent is required for substrate recognition by the erythrocyte PAF acetylhydrolase. We examined the effect of analogs of choline with successively fewer methyl groups and found that the derivative containing dimethylethanolamine inhibited PAF hydrolysis to a larger extent than the monomethyl analog. Thus, it appears that choline is preferred, but not required, in the substrate. Finally, we found that the type of linkage at the *sn*-1 position, ester or ether, did not affect the ability of the analogs to be recognized by the erythrocyte PAF acetylhydrolase. Thus, the erythrocyte PAF acetylhydrolase has marked selectivity for short acyl chains at the *sn*-2 posi-

TABLE II

Effect of PAF analogs and phosphatidylcholine on the ability of the human erythrocyte PAF acetylhydrolase to hydrolyze PAF

The PAF acetylhydrolase preparation used had a specific activity of 8.0  $\mu\text{mol/mg/h}$  at 37 °C. Each assay contained enzyme ( $4.2 \times 10^{-4}$  units), inhibitor (0–5 nmol), [acetyl- $^3\text{H}$ ]PAF (1 nmol) and 0.1 M K-Hepes (pH 7.2), in a total volume of 50  $\mu\text{l}$ . The incubations were carried out for 15 min at 37 °C.

| Additions                               | Concentration | Activity  |
|---|---------------|-----------|
|   | $\mu\text{M}$ | % control |
| 1-Palmitoyl-2-acetyl-GPC <sup>a</sup>   | 10            | 77.6      |
|   | 50            | 28.6      |
|   | 100           | 16.1      |
| 1-Hexadecyl-2-propionyl-GPC             | 10            | 92.5      |
|   | 50            | 21.7      |
|   | 100           | 22.3      |
| 1-Hexadecyl-2-butyryl-GPC               | 10            | 92.5      |
|   | 50            | 41.8      |
|   | 100           | 23.6      |
| 1-Hexadecyl-2-pentanoyl-GPC             | 10            | 103.7     |
|   | 50            | 59.0      |
|   | 100           | 28.0      |
| 1-Hexadecyl-2-hexanoyl-GPC              | 10            | 46.6      |
|   | 50            | 15.5      |
|   | 100           | 6.2       |
| 1-Hexadecyl-2-acetyl-GPDME <sup>b</sup> | 10            | 43.9      |
|   | 50            | 37.8      |
|   | 100           | 11.7      |
| 1-Hexadecyl-2-acetyl-GPMME <sup>c</sup> | 10            | 85.0      |
|   | 50            | 44.7      |
|   | 100           | 18.0      |

<sup>a</sup> GPC, glycerophosphocholine.

<sup>b</sup> DME, dimethylethanolamine.

<sup>c</sup> MME, monomethylethanolamine.

tion and prefers phosphocholine as the polar head group, a pattern like that of the plasma form of the enzyme (25).

**Oxidized Phospholipids Are Substrates for the Human Erythrocyte PAF Acetylhydrolase**—The finding that erythrocytes contain a PAF acetylhydrolase activity led us to ask if PAF was synthesized by these cells. We did not detect the synthesis of PAF by erythrocytes following treatment with calcium ionophore A23187 (data not shown). In parallel control experiments, this agonist did stimulate PAF production by neutrophils and endothelial cells. Since erythrocytes did not synthesize PAF, we considered the possibility that the role of the PAF acetylhydrolase is to catalyze the degradation of PAF synthesized and secreted by other cells. To assess this, we added radiolabeled PAF to a suspension of washed erythrocytes and measured the rate of degradation, as previously described with other cells and plasma (26). In three independent experiments, we found essentially no degradation (<3% in 15 min at 25 °C) of PAF ( $10^{-9}$  and  $10^{-7}$  M) in the absence or presence of added serum albumin (50 mg/ml). Thus, there is no indication that the PAF acetylhydrolase in intact erythrocytes comes into contact with PAF. This suggests that erythrocytes contribute to PAF degradation following lysis,

perhaps at sites of inflammations, or that the erythrocyte acetylhydrolase has additional substrates.

We found recently that phospholipids that contain an oxidatively fragmented fatty acyl residue at the *sn*-2 position are substrates for the PAF acetylhydrolase from plasma (12). The removal of these lipids, which may be toxic (8-10) or have biological activities similar to PAF (13), may be an important function for the plasma acetylhydrolase. We asked whether the PAF acetylhydrolase from erythrocytes also recognized oxidized phospholipids. We tested 1-palmitoyl-2-([1-<sup>14</sup>C]5-oxovaleroyl)-*sn*-glycero-3-phosphocholine (12) as a substrate for the purified enzyme from erythrocytes. As with the plasma enzyme, this model oxidized phospholipid was hydrolyzed by the erythrocyte acetylhydrolase. The reaction was dependent on time and enzyme concentration. The addition of substrate at levels above 6  $\mu$ M resulted in inhibition of hydrolysis, suggesting that the accumulation of products of the reaction inhibit the enzyme. Thus, it was not possible to quantitatively compare the rates of the reaction using saturating concentrations of PAF and the above oxidized phospholipid substrate. To circumvent this, we tested another oxidized phospholipid, 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine and found that it was hydrolyzed by the purified enzyme at a rate equivalent to 53% of that at which PAF was hydrolyzed. Thus, the substrate specificity of the erythrocyte PAF acetylhydrolase is similar to that of the human plasma PAF acetylhydrolase since both activities hydrolyze oxidatively fragmented phospholipids as well as PAF.

It was possible that our purified preparation contained two activities, the PAF acetylhydrolase and another that catalyzed the degradation of oxidized phospholipids. To determine whether the hydrolyses of PAF and oxidized phospholipids were catalyzed by the same activity, we tested the effect of compounds that inhibit the hydrolysis of PAF by erythrocyte acetylhydrolase on the hydrolysis of oxidized phospholipids. We found (Fig. 3) that both activities were stimulated by DTE and inhibited by 5,5'-dithiobis(2-nitrobenzoic acid), and NaF and to approximately the same extent. These results support the conclusion that the activity against different substrates is due to a single enzyme, the PAF acetylhydrolase.

**Kinetic Properties of the PAF Acetylhydrolase from Erythrocytes**—The dependence of enzyme activity on substrate

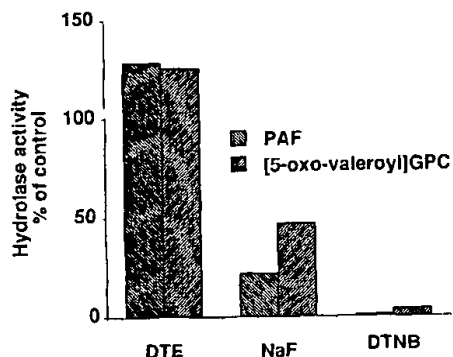


FIG. 3. The activities that hydrolyze PAF and 1-palmitoyl-2[5-oxovaleroyl]glycerophosphocholine in human erythrocytes are affected by the same compounds. A human erythrocyte lysate (1  $\mu$ l of a preparation containing 0.5 unit/ml of PAF acetylhydrolase activity) was incubated in 0.1 M K-Hepes buffer (pH 7.2) with substrate (62.5  $\mu$ M) and DTE (19 mM), NaF (19 mM), or 5,5'-dithiobis(2-nitrobenzoic acid) (1.9 mM), in a total volume of 40  $\mu$ l, for 30 min at 37 °C. The products of the reaction were separated from starting substrate, as described (25). Results are representative of at least two independent experiments. GPC, glycerophosphocholine.

concentration is shown in Fig. 4. The  $K_m$  for PAF was determined to be 12.5  $\mu$ M. Most phospholipases display surface dilution kinetics, a phenomenon that indicates that the enzyme prefers substrates associated with structures over free monomers. Such enzymes recognize the abundance of a particular lipid on the surface of a micelle or membrane, rather than the absolute concentration in the assay (35). Under these conditions, adding detergent to the assay has opposing effects depending on the substrate concentration. At concentrations of substrate below the critical micellar concentration (reported to be 2.5  $\mu$ M for PAF, Ref. 16), the addition of Triton X-100 should increase the rate of hydrolysis by forming mixed micelles of substrate and detergent. This was the result we obtained (Fig. 5) with the purified erythrocyte enzyme. In contrast, at PAF concentrations above the critical micellar concentration, the addition of Triton X-100 resulted in inhibition, presumably by decreasing the abundance ("diluting") of the substrate on the surface of a given micelle. These results suggest that the enzyme prefers micellar, as opposed to monomeric, substrates. This hypothesis was confirmed in an experiment in which the concentration of Triton X-100 alone or Triton X-100 and PAF were increased proportionately. In the first case, the substrate concentration at the surface of the micelles was diluted by increasing the number of micelles among which the fixed amount of substrate was randomly distributed. This resulted in inhibition (Fig. 6A).

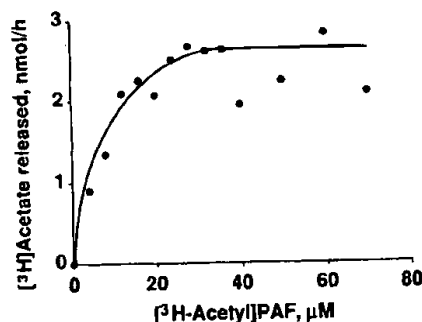


FIG. 4. Kinetics of the human erythrocyte PAF acetylhydrolase. A purified preparation of the human erythrocyte PAF acetylhydrolase (10  $\mu$ l) containing 0.05 unit/ml of activity in 0.1 M K-Hepes buffer (pH 7.2), was mixed with [acetyl-<sup>3</sup>H]PAF (0-80  $\mu$ M) in a total volume of 50  $\mu$ l. Incubations were performed at 37 °C for 15 min. The product of the reaction was separated from starting substrate, as described (25).

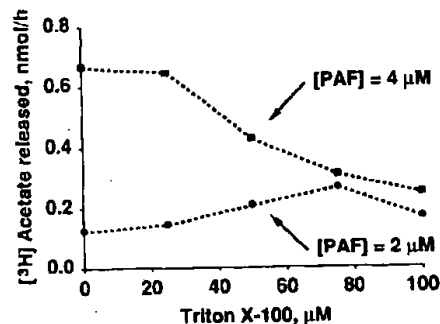


FIG. 5. Effect of Triton X-100 on catalytic activity at PAF concentrations below (2  $\mu$ M) and above (4  $\mu$ M) the critical micellar concentration of PAF. A purified preparation of the human erythrocyte PAF acetylhydrolase (10  $\mu$ l) containing 0.05 unit/ml of activity was mixed with [acetyl-<sup>3</sup>H]PAF (2 or 4  $\mu$ M) and Triton X-100 (0-100  $\mu$ M), in a total volume of 50  $\mu$ l. Incubations were continued as described (25).

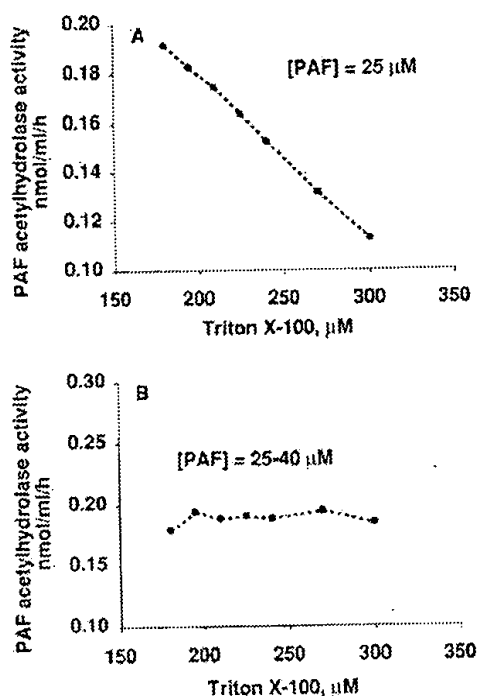


FIG. 6. A, effect of increasing Triton X-100 at constant PAF concentrations. A purified preparation of the human erythrocyte PAF acetylhydrolase (10 μl) containing 0.42 unit/ml of activity was mixed with [ $^3$ H]PAF (24 μM) and Triton X-100 (180–300 μM), in a total volume of 50 μl. Incubations were continued as described (25). B, effect of increasing Triton X-100 and PAF at a constant ratio. A purified preparation of the human erythrocyte PAF acetylhydrolase (10 μl) containing 0.42 unit/ml of activity was mixed with [ $^3$ H] PAF (24–40 μM) and Triton X-100 (180–300 μM), in a total volume of 50 μl. Incubations were continued as described (25).

In the second part of the experiment, both substrate and free micelles were increased proportionately, so that the substrate abundance at the micellar surface remained constant. This resulted in no effect on the enzymatic activity even though the absolute concentration of PAF was increased over non-saturating concentrations (Fig. 6B). From these results we concluded that the erythrocyte PAF acetylhydrolase, even though it is a soluble enzyme, exhibits surface dilution kinetics.

**Characterization of the Two Proteins in the Purified Preparation.**—We separated the proteins by subjecting the preparation to SDS-PAGE and then recovered the individual components by slicing the gel and incubating the slices with buffer. The separated proteins after recovery from the gel are shown in Fig. 7 (lanes 10 and 11). Polyclonal antibodies against each of the two proteins were generated in New Zealand White rabbits, as described under "Experimental Procedures." The antibodies recognized primarily the antigen that was used to elicit its generation (Fig. 8). However, there was some cross-reactivity of the anti-25-kDa antibody with the 31-kDa protein and of the anti-31-kDa antibody with the 25-kDa protein, suggesting either that the separation of the two proteins had not been complete or that they were related, perhaps as a precursor and product of proteolysis. The antibodies then were tested for their ability to precipitate PAF acetylhydrolase activity. We found (Fig. 9) that the antibody raised against the 25-kDa protein immunoprecipitated PAF acetylhydrolase activity from a partially purified preparation of the enzyme, suggesting that the 25-kDa protein is the enzyme. This also suggested that the 31-kDa component was

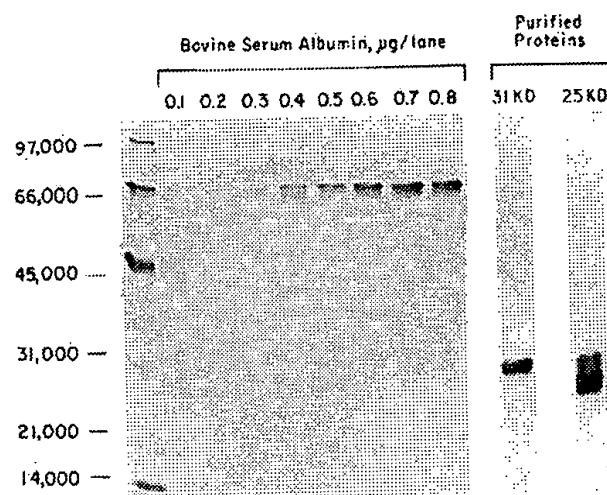


FIG. 7. Separation of 25-kDa and 31-kDa protein components by SDS-PAGE. Lane 1, molecular weight markers; lanes 2–9 contained bovine serum albumin (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 μg) used as a calibration standard to determine the amount of protein detected by silver staining; lane 10, 3.5 μg of 31-kDa protein component; lane 11, 3.5 μg of 25-kDa protein component.

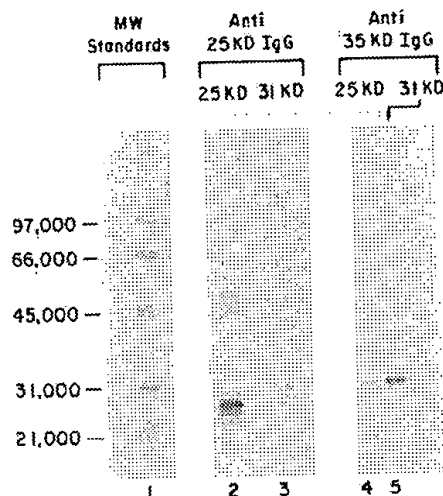


FIG. 8. Western blot of 25-kDa and 31-kDa proteins with polyclonal antibodies generated against the purified products. Lane 1, molecular weight markers; lanes 2 and 4, 3.5 μg of the 25-kDa protein; lanes 3 and 5, 3.5 μg of the 31-kDa protein. Lane 1 was stained with Amido Black; lanes 2 and 3 were probed with the rabbit antibody raised against the 25-kDa protein and developed with alkaline phosphatase-labeled anti-rabbit IgG; lanes 4 and 5 were probed with the rabbit antibody raised against the 31-kDa protein and developed with alkaline phosphatase-labeled anti-rabbit IgG.

not the enzyme and that it was not related to the 25-kDa component through a proteolytic event.

We separated the two proteins by SDS-PAGE, eluted them from the gel as above, and sequenced them. We found that the amino-terminal residue of the band at 31,000 Da was blocked. We therefore treated it with CNBr, performed SDS-PAGE again, transferred the reaction mixture to a polyvinylidene difluoride membrane, and carried out microsequencing, as described under "Experimental Procedures." The sequence of the 11 amino-terminal residues of the most abundant fragment obtained was: (M)ALNHGEEQVRL. A search using a protein sequence data bank indicated that this sequence was a perfect match with the human 2,3-bisphosphoglycerate mu-

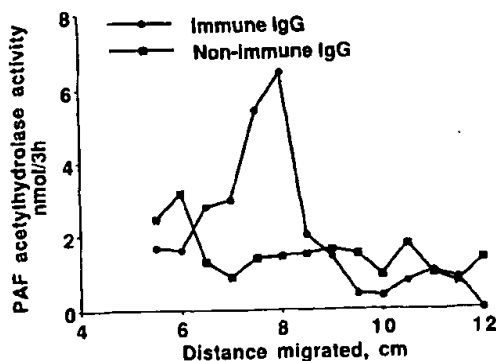


FIG. 9. Immunoprecipitation of PAF acetylhydrolase activity by the antibody raised against the 25-kDa protein component. The effluent from the native polyacrylamide gel electrophoresis step (100  $\mu$ l) was preincubated for 16 h at 4 °C with preimmune rabbit IgG or with the antibody raised against the 25-kDa protein, in a total volume of 500  $\mu$ l. The mixtures were treated with 100  $\mu$ l of a 50:50 suspension of protein A-Sepharose in phosphate-buffered saline to bind the immune complexes. The beads were then washed and subjected to native polyacrylamide gel electrophoresis. Each lane was sliced and assayed for PAF acetylhydrolase activity.

tase (EC 5.4.2.4), which is present in high amounts in erythrocytes. The substrate specificity of the purified PAF acetylhydrolase indicated that it was unlikely that 2,3-bisphosphoglycerate mutase and the PAF acetylhydrolase were two activities of a single enzyme. However, this was tested using our purified preparation (containing both the 25- and 31-kDa proteins) in the following manner. First, we performed a temperature inactivation experiment and found that PAF acetylhydrolase was more susceptible than the glyceromutase activity (Fig. 10). Second, we tested the effect of NaF (5 and 50 mM) on each activity since we had shown (Fig. 3) that it inhibited the acetylhydrolase activity. In this experiment, the two concentrations of NaF inhibited PAF acetylhydrolase activity by 24 and 64%, respectively. In contrast, the glyceromutase activity in the preparation was resistant to the treatment (99% and 90% of control activity remaining, respectively). Third, we showed that 2,3-diphosphoglycerate (2 mM and 20 mM) inhibited glyceromutase activity (10 and 63%, respectively), but found no effect on PAF acetylhydrolase activity (101 and 105% of control activity remaining, respectively). Finally, NaHSO<sub>3</sub> inhibited PAF acetylhydrolase activity at 5 and 50 mM (31 and 85%, respectively), while the glyceromutase was more resistant to this treatment (107 and 62% of control activity remaining). Thus, we concluded that the protein migrating with an apparent molecular weight of 31,000 was 2,3-bisphosphoglycerate mutase, and that it did not have PAF acetylhydrolase activity.

We next sequenced the 25,000-Da component and found that the sequence of the 10 amino-terminal residues was: (M)KPLVVFVILGG. There were no perfect matches of this stretch of amino acids with sequences present in the data bank. However, this sequence has identity (82%) with a stretch of amino acids present in cytidylate kinase (EC 2.7.4.14) and adenylate kinase (EC 2.7.4.3, 45% identity). We conclude that this protein is pure and that this sequence likely represents the amino terminus of the PAF acetylhydrolase from human erythrocytes.

**The Erythrocyte PAF Acetylhydrolase Is Not a Previously Described Enzyme**—Several lines of evidence indicated that the PAF acetylhydrolase in erythrocytes is a novel, uncharacterized activity. However, we examined the possibility that it was due to contaminating plasma PAF acetylhydrolase. We

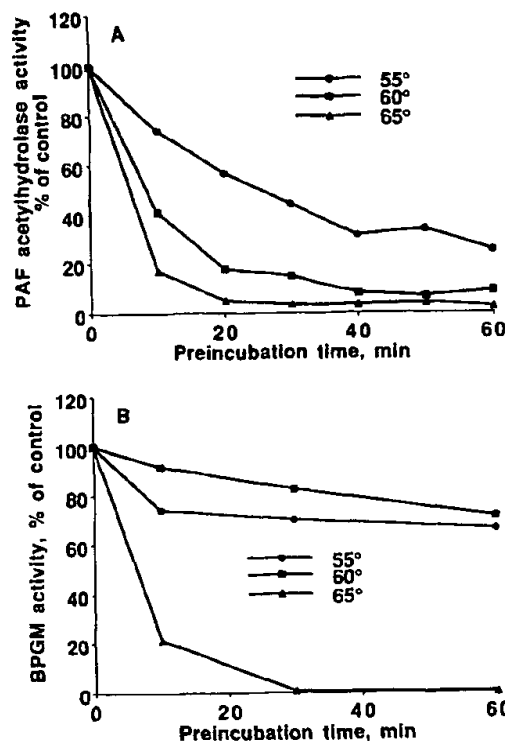


FIG. 10. Temperature inactivation of PAF acetylhydrolase activity (A) and bisphosphoglycerate mutase activity (B) in a partially purified preparation. A purified preparation of human erythrocyte PAF acetylhydrolase (50  $\mu$ l of a preparation containing 0.11 unit of activity per ml) was incubated at the temperatures indicated for 0–60 min. Then, 20- $\mu$ l aliquots were removed and assayed for PAF acetylhydrolase and bisphosphoglycerate mutase activities.

found that the plasma and erythrocyte activities, while sharing the same substrate specificity, could be distinguished by several criteria. The erythrocyte activity was inhibited by sodium fluoride, *p*-bromophenacyl bromide, and diethylpyrocarbonate, and it was highly sensitive to proteolysis. In contrast, the plasma activity was unaffected by these treatments (21, 30). Further, we found that the erythrocyte PAF acetylhydrolase was stimulated by dithiothreitol, dithioerythritol, and glutathione and was inhibited by 5,5'-dithiobis-(2-nitrobenzoic acid) and iodoacetate. None of these agents affect the human plasma PAF acetylhydrolase (30). From these results, we concluded that the erythrocyte, but not the plasma, form of PAF acetylhydrolase contains an essential sulfhydryl moiety. Finally, the serine esterase inhibitors diisopropyl fluorophosphate (30) and *p*-nitroguanidinobenzoate (data not shown) inhibited PAF hydrolysis by the plasma enzyme to a much lesser extent than the erythrocyte activity. Thus, the PAF acetylhydrolase activity found in erythrocytes was clearly not due to contaminating plasma.

We next considered that erythrocyte acetylcholinesterase might catalyze PAF hydrolysis. We examined the effect of acetylcholine on PAF hydrolysis, using a partially purified preparation of the erythrocyte PAF acetylhydrolase. Acetylcholine did not inhibit PAF hydrolysis at the concentrations tested (40  $\mu$ M–1 mM), even though there was a marked molar excess of inhibitor in relation to substrate. Esterase D was excluded as the catalyst for PAF hydrolysis by an analogous experiment: we added 4-methylumbelliferyl acetate, a compound used as the substrate to purify esterase D, to assays and found that it did not inhibit PAF hydrolysis at three



different concentrations of PAF and at large excesses compared to substrate. A third candidate was the phospholipase A described in human erythrocytes by Paysant *et al.* (36), which utilizes phosphatidylcholines and phosphatidylethanolamines as substrates and requires pretreatment with trypsin for maximal activity. In contrast, trypsin inhibited the erythrocyte PAF acetylhydrolase, which does not utilize phosphatidylcholine as substrate (30). The addition of calcium, an obligatory cofactor for activity of most phospholipases A<sub>2</sub>, is not necessary for PAF hydrolysis by the erythrocyte activity, and the divalent metal chelator EDTA does not significantly inhibit PAF hydrolysis (30). Finally, we considered the possibility that the activity was a nonspecific one that was capable of removing an acetyl group from aliphatic esters. Therefore, we supplemented standard assays with N-acetyllysine and with tributyrin. These agents did not inhibit PAF hydrolysis, suggesting that a phosphatidylglycerol backbone is a requirement for substrate recognition. From these results, we concluded that the PAF acetylhydrolase activity measured in human erythrocytes is a novel phospholipase, perhaps related to the plasma enzyme.

#### DISCUSSION

We have purified the erythrocyte PAF acetylhydrolase 15,600-fold. The product of this purification protocol has a specific activity of 48.3  $\mu\text{mol/h/mg}$  of protein, and initially we recovered 7% of the activity present in erythrocyte lysates. The biggest loss of activity occurred during native polyacrylamide gel electrophoresis. We subsequently found that the recovery during this step can be significantly improved if electrophoresis is performed in the presence of a reducing agent. The product of the purification contains two main protein bands of apparent molecular weights 31,000 and 25,000.

Several lines of evidence suggest that the 25-kDa protein is the PAF acetylhydrolase. First, we sequenced the 31-kDa protein and found that it was an identical match with the human erythrocyte bisphosphoglycerate mutase. This enzyme did not account for the PAF acetylhydrolase activity in our preparation, since the two activities had different temperature stabilities and were inhibited by different agents. Second, the intensity of staining of the 25-kDa protein component correlates with activity during purification. Third, an antibody raised against the 25-kDa protein component precipitates PAF acetylhydrolase activity.

The human erythrocyte PAF acetylhydrolase is calcium independent and has a substrate specificity similar to that of the human plasma PAF acetylhydrolase. It recognizes phosphoglycerides with *sn*-1 alkyl and *sn*-1 acyl groups with the same affinity. Likewise, the type of *sn*-3 head group does not markedly affect the ability of a substrate to be recognized by the enzyme. However, the erythrocyte PAF acetylhydrolase has an absolute requirement for a short acyl chain at the *sn*-2 position. Like the human plasma activity, the erythrocyte PAF acetylhydrolase can also hydrolyze phospholipids that contain short *sn*-2 acyl residues generated by oxidative fragmentation of polyunsaturated residues. This may be an important role for the enzyme since we found that human erythrocytes do not synthesize PAF nor do they efficiently degrade exogenous PAF. However, they are likely to generate a myriad of oxidized phospholipids during their life span, due to the oxidant stress to which they are subjected. The peroxidation of lipids is enhanced in the presence of hemoglobin and high oxygen tensions, a combination frequently encountered by erythrocytes. The observation that the enzyme exhibits surface dilution kinetics is consistent with the hypoth-

esis that oxidatively fragmented phospholipids are substrates of the erythrocyte PAF acetylhydrolase *in vivo*. This observation indicates that hydrolysis occurs preferentially at the membrane interface, which is the primary site for the generation of oxidized phospholipids. Interestingly, Yoshida *et al.* (31) reported that 0.2% of the total erythrocyte PAF acetylhydrolase activity is membrane-bound, suggesting that the enzyme may associate with the membrane to hydrolyze phospholipid substrates, while the bulk of activity remains in the cytosol.

The erythrocyte PAF acetylhydrolase is sensitive to oxidation. First, the enzyme requires the addition of reducing agents for maximal activity. Second, the activity is inhibited by heavy metals such as lead, cadmium, and copper (21). These results suggest that the enzyme has a sulfhydryl group(s) essential for activity. The fact that this enzyme is likely involved in the removal of products of lipid peroxidation reactions and is itself sensitive to oxidation suggests that the balance between enzyme activity and oxidative state of the cell may contribute to define cell homeostasis and viability.

The possibility that other previously characterized erythrocyte activities could account for PAF hydrolysis was considered. A variety of esterases (acetylcholinesterase, tributyrin esterase, nonspecific esterase, esterase D, and others) were ruled out as candidates that could account for the PAF acetylhydrolase activity. Likewise, the 2,3-bisphosphoglycerate mutase activity present in our preparation did not account for the PAF acetylhydrolase activity.

In summary, we have purified and characterized the PAF acetylhydrolase from human erythrocytes. The function of this activity *in vivo* is likely to be the hydrolysis of the phospholipid products of oxidative fragmentation of membrane phospholipids. This hydrolysis step serves at least three purposes. First, it allows subsequent restoration of membrane integrity by reacylation of the lyso derivatives with long chain fatty acyl groups. Second, it hinders further oxidative reactions by scavenging active phospholipid species that may themselves enhance lipid peroxidation. Finally, it degrades oxidatively fragmented phospholipids which, when produced in large amounts, can be toxic or have various pathological actions. In fact, work by Kobayashi *et al.* (37) and by Inoue *et al.* (38) suggests that cytotoxic phospholipids are generated during peroxidation catalyzed by oxyhemoglobin and that this process induces the damage of erythrocyte membranes.

In addition to its role as a scavenger of oxidatively fragmented phospholipids, the acetylhydrolase may participate in PAF metabolism under some circumstances. For example, hemolysis is common in inflammatory conditions, particularly those in which PAF has been implicated as a mediator, and lysis of the erythrocyte could help turn off the inflammatory signal by speeding the hydrolysis of PAF. In fact, van Asbeck *et al.* (39) showed that erythrocytes limited inflammation in a model of lung injury (39). They attributed the effect to scavenging of oxygen radicals but did not test whether hydrolysis of PAF or oxidized phospholipids was a feature of the response.

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